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The role of lichenized algae in the production of *Cladonia verticillaris* depsidones, revealed by using alginate-immobilized cells

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ABSTRACT

The mycobiont of the lichen *Cladonia verticillaris* produces two depsidones, protocetraric and fumarprotocetraric acids, in the nature. This last compound is produced from the former depsidone. To study the role of the algal partner in the production of these depsidones in the lichen thallus, fungal and algal partners were separated and immobilized in calcium alginate. The fungal immobilizates, as lichenized or isolated mycobiont, produced both depsidones, preferently fumarprotocetraric acid from acetate as a precursor. However, when algal immobilizates were co-incubated on acetate with the fungal ones, protocetraric acid was over-produced during the first 11 days of incubation to be later converted into fumarprotocetraric acid. We hypothesized about the algal production of a diffusible metabolite that inhibits the transformation of a depsidone into the other one. © 2016 Trade Science Inc. - INDIA

KEYWORDS

Alginate;
Cladonia verticillaris;
Immobilizates;
Fumarprotocetraric acid;
Protocetraric acid.

INTRODUCTION

Lichens are intimate and long-term specific symbioses of photosynthetic algae or cyanobacteria and heterotrophic fungi joined to form a new biological entity different from its individual components^[1]. The symbiosis state makes possible the production of several families of organic compounds, secondary metabolites developing varied biological roles. Between these compounds, the most well-known are those phenols from the acetate–polymalonate pathway, which are produced by the mycobiont, secreted outside the fungal hyphae and crystallized on the cortex to form a protective screen against UV radi-

tion^[2].

Cladonia verticillaris is chemically characterized by the production of two depsidones, protocetraric and fumarprotocetraric acids, and a depside, atranorin, precursor of the two depsidones. These three compounds derive from methyl-3-orsellinate produced by the action of a polyketide synthase (PKS).

Polyketide synthases are a family of enzymes or enzyme complexes that produce polyketides, a large class of secondary metabolites in bacteria, fungi, plants, and a few animals lineages^[3]. Each polyketide-synthase consists of several domains developing specific, catalytic functions, separated by

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short spacer regions. From N- to C-terminus, domains are arranged as: acyltransferase (AT), acyl carrier protein (ACP), keto-synthase (KS), ketoreductase (KR), dehydratase (DH), enoylreductase (ER), O- or C- methyltransferases (MT), sulfhydrolase (SH) and thioesterase (TE). Further optional accessory domains are represented by cy-

class (CYC)^[4] and methyl transferase (MT)^[5] activities. These enzymes assemble structurally diverse products from simple acyl-CoA substrates by using a catalytic cycle involving decarboxylative Claisen condensations and variable modifications, such as reduction and dehydration. The occurrence of the Claisen condensation is the main step in polyketide

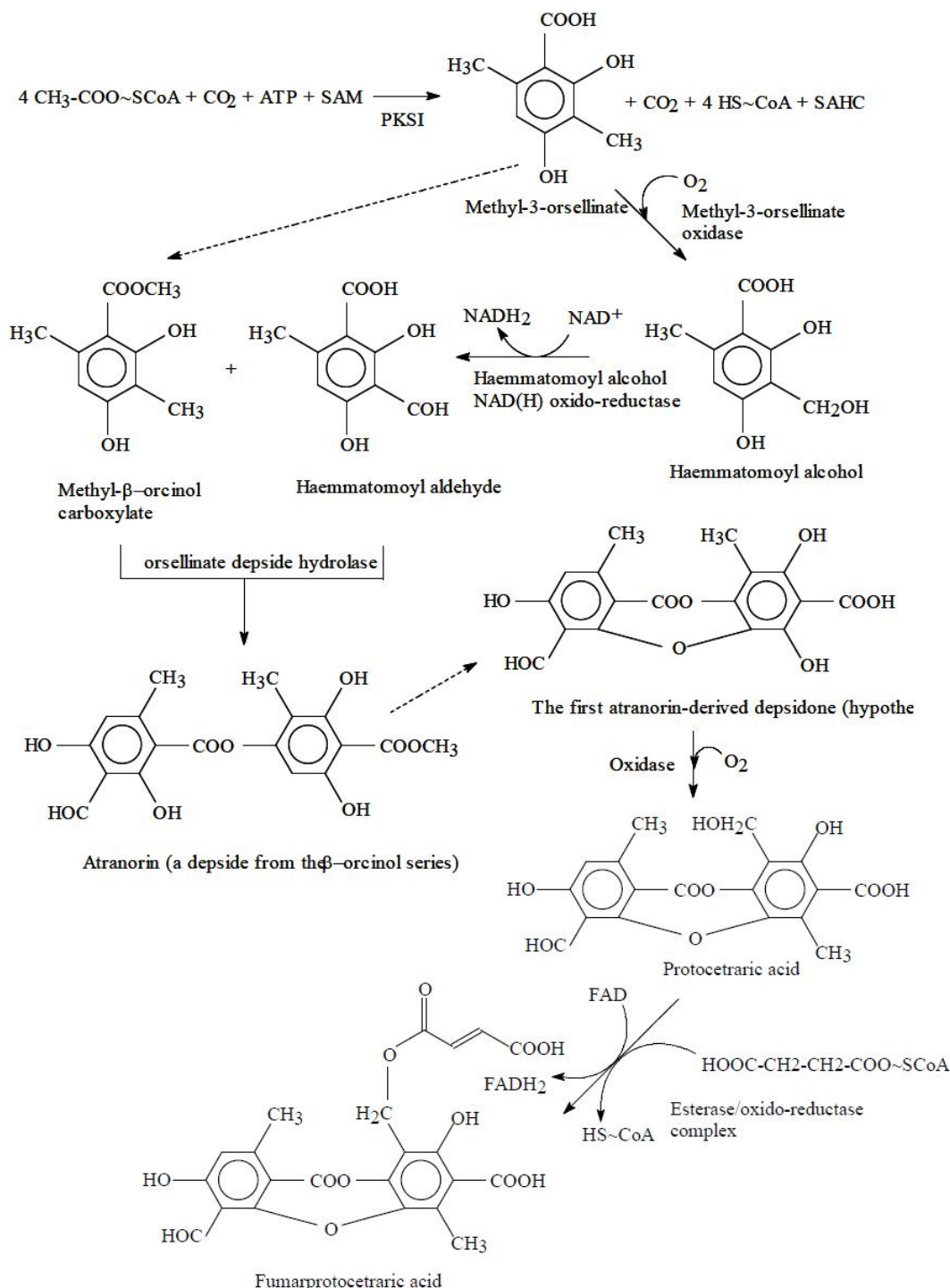


Figure 1 : Metabolic pathway of depsidone production by *Cladonia verticillaris*.

biosynthesis^[6] since it permits the production of a phenolic acid with a methyl group as substituent, such as orsellinic acid or 6-methylsalicylate, whereas the absence of this condensation reaction only produces polihydroxy derivatives such as tetrahydroxynaphthalene. After Claisen condensation, a new acetyl-CoA molecule binds on KS subunit to be newly transferred on elongating chain to form a final, lineal precursor of 8C before cyclisation. Products of PKS1 action are orsellinic acid or methyl-3-orsellinate when PKS contains an intrinsic methyl transferase subunit (Figure 1), although many derivatives can be synthesized by introducing into orsellinate molecule several chemical motifs through post-PKS modifications defined as tailoring reactions by Rawlings^[7].

Methyl-3-orsellinate is modified to haematomoyl aldehyde through to successive oxidations^[8]. Both phenols are then esterified by an orsellinate depside hydrolase^[9] to produce the depside, atranorin (Figure 1). The natural occurrence of natural depside-depsidone pairs strongly suggests that depsidone could be produced by a single dehydration of the depside. However, Armaleo et al.^[10] described the first lichen PKS cluster likely to be implicated in the biosynthesis of a depside and the corresponding depsidone. Among the many PKS genes in *Cladonia grayi*, they are four, named CgrPKS13 to 16, potentially responsible of the biosynthesis of the depsidone grayanic acid. In the *C. grayi* genome, CgrPKS16 clustered with a CytP450 and an O-methyltransferase gene, appropriately matching the three compounds in the grayanic acid pathway. Specifically, the authors propose that a single PKS synthesizes two aromatic rings and links them into a depside, and the depside-to-depsidone transition requires only a cytochrome P450.

The main enzymes involved in the phenols production are synthesized exclusively by the fungal partner. This excludes the alga of the bionthesys of the orsellinate derivatives. However, Culbertson and Ahmadjian^[11] propose that the algal partner is able to regulate fungal phenol production by the action of effectors secreted to the intercellular spaces.

The aim of this work attempt to reveal, by means of cell immobilization techniques, if the alga really

regulates the synthesis of these phenols or only plays a nutritional role, required to maintain the size of the fugal pool of acetyl-CoA necessary to support the fungal respiration and the synthesis of secondary metabolites.

MATERIAL AND METHODS

Biological material

C. verticillaris (Raddi) Fr., an endemic lichen species of the Brazilian littoral and of the tabuleiros of the interior of the north-east of Brazil, was used throughout this work. Lichens grow on quartzarenic neosols that have low content of organic matter, low capacity to retain water and nutrients, low cation exchange capacity, low base saturation, increased acidity with the depth, sandy texture, predominance of kaolinite in the clay fraction and a fragile, physical structure^[12].

The samples were collected in the foothills of Serra da Prata, 20 km to the north of the municipality of Saloá (8° 57' 23" S, 36° 43' 22" W), to 273 km from Recife, in an open field at 10 km east from the road PE 223. Thalli were harvested from a unique plot of 5 m², continuously exposed to sunshine without shade of any vegetation. We chose those specimens that had developed seven whorls and stored them in paper boxes at 20 °C ± 2 °C, in the dark, until required. All the samples were collected from a unique environment to avoid changes in the concentration of bioactive compounds derived from the different soils or exposure degrees.

The material was identified and the voucher specimen deposited in the Herbarium UFP of the Department of Botany of Pernambuco's Federal University, with the record number 52.299. *Parmotrema dilatatum* (Vainio) Pulls, used for the extraction of protocetraric acid (PRO), was collected at the same place, its record number being 39.893.

Bionts isolation and immobilization

Bionts were isolated from thalli of *C. verticillaris* according to Fontaniella et al^[13]. Sufficiently rehydrated thalli (0.5 g) were gently macerated in a mortar with 10 mL of distilled water. Homogenates were filtered through six layers of

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cheese-cloth and both myco and phycobionts were separated by centrifugation in sucrose: potassium iodide and phosphate:sucrose gradients, as described.

Mechanically disgregated thalli (0.5 g), or isolated chloro- or mycobionts (0.2 g) were dispersed in 10 mL 2% (w/v) sodium alginate and the different cell suspensions were added drop-to-drop, by means of a syringe, on 50 mL of a 20 mM calcium chloride solution. After 24 h, the calcium alginate spheres (immobilisates) were ready for their use. The beads of calcium alginate were supplemented with 50 mL 1.0 mM sodium acetate and maintained, gently stirred, at 22 °C for 20 days in the light of a photon flux rate of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. An aliquot of 5.0 mL from bath solution was collected at different times to extract lichen phenolics and replaced with the same volume of fresh medium.

Depsidones extraction and quantitation

Phenolics were extracted by mixing the aliquot of the bath solution with 5 mL diethyl ether:ethyl acetate (65:35 v/v). The mixture was vigorously stirred and the organic phase separated with a micropipette. Aqueous phase was then extracted with 5 mL chloroform:acetone (50:50 v/v), stirred for 5 min and the organic phase recovered and mixed with the first one. Organic extracts were dried in air-flow. The residue was redissolved in 2-0 mL methanol and analysed by HPLC according to Feige et al.^[14] using a Varian 5060 liquid chromatograph equipped with a Vista CDS 415 computer. A reverse phase MCH-10, 5 μm particle diameter, 250 mm \times 4.6 mm column was used. Two solvent systems were employed. System A was MilliQ water containing 1% (v/v) ortho-phosphoric acid and system B was 100% methanol. The run started with 30% B and continued isocratically for 1 min at 0.7 mL min⁻¹. After this, 10 μL was injected and the solvent B was increased to 70% within 14 min, then up to 100% in 30 min, and then isocratically in 100% B for a further 8 min. At the end of the run, system B was decreased to 30% within 1 min and the column flushed with 30% B for 16 min before a new run was started. Detection was carried out by a UV set at 254 nm.

Protocetraric acid, purified from *P. dilatatum*

thalli (Vain.) and fumarprotocetraric acid and atranorin from *C. verticillaris* thallus in the Laboratory of Natural Products of the Department of Biochemistry of Pernambuco's Federal University, Recife, Brazil,^[15] were used as standards. Commercial atranorin (Sigma) was also used with the same purpose. The amount of protocetraric and fumarprotocetraric acids recovered from the medium is referred to the mass of immobilized thallus, 0.5 g of lichen tissue, or 0.2 pf each one of the bionts, by each bioreactor.

Light microscopy

Sections of immobilisates of about 8 μm thickness were obtained by using a freezing microtome and observed using a Zeiss invertomicroscope. Photographs were obtained by a Coolpix 5000 digital camera from Nikon. Natural fluorescence of chlorophylls, indicating the position of the chlorobionts, was observed using an Olympus DP50 fluorescence microscope adapted with an Olympus DP72 camera. Relative areas of both bionts inside the thallus was calculated by using an image analysis program, Image-Tool 2.0.

RESULTS AND DISCUSSION

The relative areas occupied for chloro- and micobiontes in *C. verticillaris* thalli were measured by image analysis of semi-thin cuts of podetia (Figure 2A and B) as well as of scyphi (Figure 1C). Values were obtained using preferably the visualizations in florescence microscopy. The podetia of *C. verticillaris* consist of 90,8 % fungi and 9,2 % algae. Practically identical results were obtained for the scyphi. These values (per cent of each one biont in the whole thallus) facilitate the estimation of the loss of phenols recovering during the process of separation of the mycobiont, as it is shown in the TABLE 1.

Atranorin was found neither in the intact thallus nor in the isolated mycobiont by HPLC analyses. It is also necessary herein to emphasize that protocetraric acid concentration in both intact lichen thallus and isolated mycobiont at zero time, before immobilization, was higher than that of

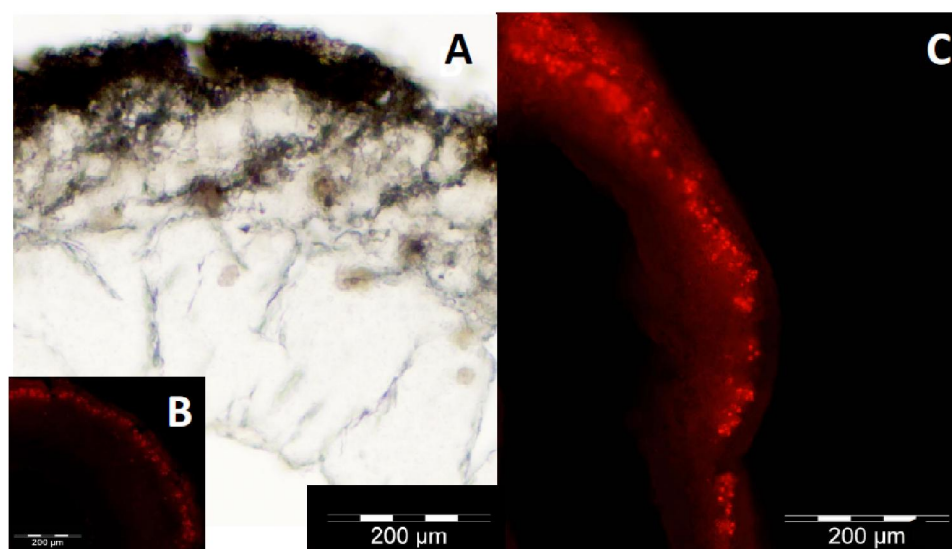


Figure 2 : Observation of semi-thin cross sections of *C. verticillaris* podetium by light (A) or fluorescence (B) microscope. Algal layer is observed as green cells in A or by red chlorophyll autofluorescence in B. C) Cross section of *C. verticillaris* scyphi showing fluorescent algal cells

TABLE 1 : Initial content of both protocetraric and fumarprotocetraric acids in *C. verticillaris* thallus and its isolated mycobiont

Material	Protocetraric acid (mg g ⁻¹ fresh weight)		Fumarprotocetraric acid (mg g ⁻¹ fresh weight)	
	Observed	Expected	Observed	Expected
Lichen thallus	106.52 ± 9.71		44.19 ± 3.98	
Isolated mycobiont	71.07 ± 6.54	96.72 ± 9.33±	31.88 ± 3.01	40.12 ± 3.94

fumarprotocetraric acid (TABLE 1) whereas this last depsidone was secreted from the immobilisates the media in an amount highest than that of its depsidone precursor (Figure 3). During the six first days of immobilisate. incubation on acetate, secretion of protocetraric acid decreased whereas that of fumarprotocetraric acids increased in an inverse way. This suggested that both depsidones could be transformed one in the other one. Esterification of protocetraric acid with succinyl-CoA coupled to a redox reaction was found for *C. verticillaris* by Fontaniella et al.^[16]. The reverse reaction could be catalyzed by the same esterase but fumaric acid instead of succinic acid might be produced after hydrolysis.

The fungal enzyme that produced fumarprotocetraric from protocetraric acid depends on the supply of succinyl-CoA, or algal photoassimilates that will be oxidized by the mycobiont, and an oxidant (FAD, FMN). For this reason, the production and secretion of the

depsidones drastically diminished from fungal immobilisates in the absence of the algal partner (Figure 4). *C. verticillaris* develops enzymatic activities in the light higher than in the dark^[17]. This could be in agreement with the requirement of the photochemical activity of the chlorobionts to maintain the pool of acetyl-CoA.

Nevertheless, when individual immobilisates of isolated myco- and chlorobionts were incubated together on 1.0 mM sodium acetate, the time-course of depsidone secretion of dramatically changed. Protocetraric acid was secreted at very high amounts to the media, 20 times higher than that found for immobilisates of whole thallus, whereas the amount of fumarprotocetraric recovered from the media remained unchanged or slightly decreased during the first 11 days of incubation. From this time, fumarprotocetraric acid linearly increased in the media whereas protocetraric acid linearly decreased (Figure 5). This could be interpreted as a new proof about the catalytic conversion of protocetraric acid

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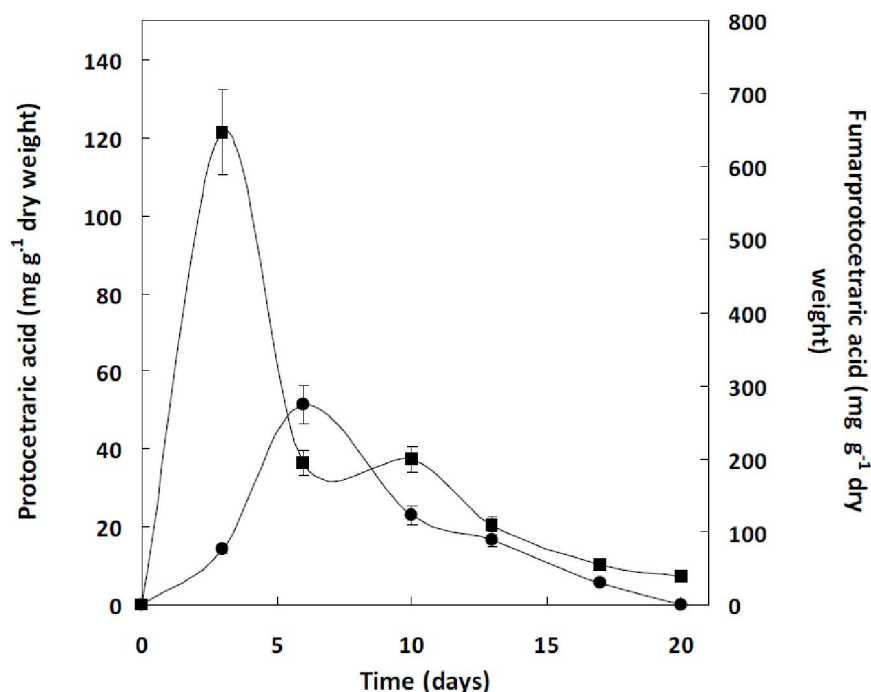


Figure 3 : Time-course of both protocetraric (●) and fumarprotocetraric (■) acids by alginate-immobilized lichen thalli. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols

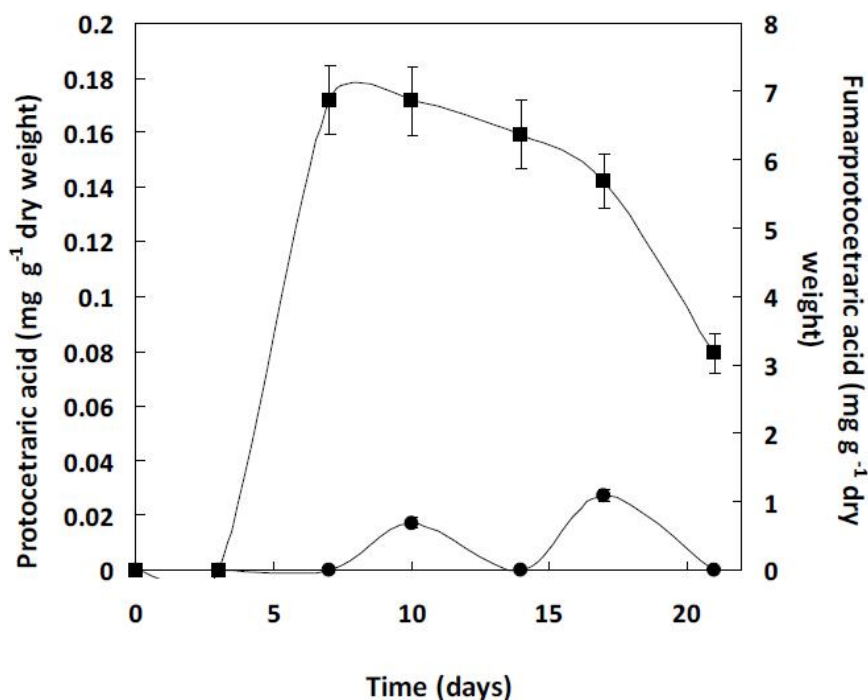


Figure 4 : Time-course of both protocetraric (●) and fumarprotocetraric (■) acids by alginate-immobilized mycobionts isolated from lichen thalli. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols

into fumarprotocetraric acid.

If the hypothesis of Culberson and Ahmadjian^[11] is correct, there is only a possible explanation for the results obtained here. The transformation between

both depsidones was possible in fungal immobilisates since they mainly synthesized fumarprotocetraric acid, though in small amounts, whereas protocetraric acid quantity was kept low

and constant (Figure 4). This could be due to the supplement of photoassimilates provided by immobilized algae that photosynthesized without the inhibitory restrictions imposed by the direct contact with the fungal partner^[18]. This process could be carried out in absence of a metabolite of algal origin that could disable the oxidative esterification of the protocetraric acid.

However, the accumulation in the media of protocetraric acid during the first 11 days of experimentation when immobilisates of newly isolated algae were incubated together fungal immobilisates should relate to the inability of fungal hyphae for synthesize fumarprotocetraric acid due to the secretion by immobilized algal cells of the alga and its capture by the fungal hyphae of an inhibitor of the

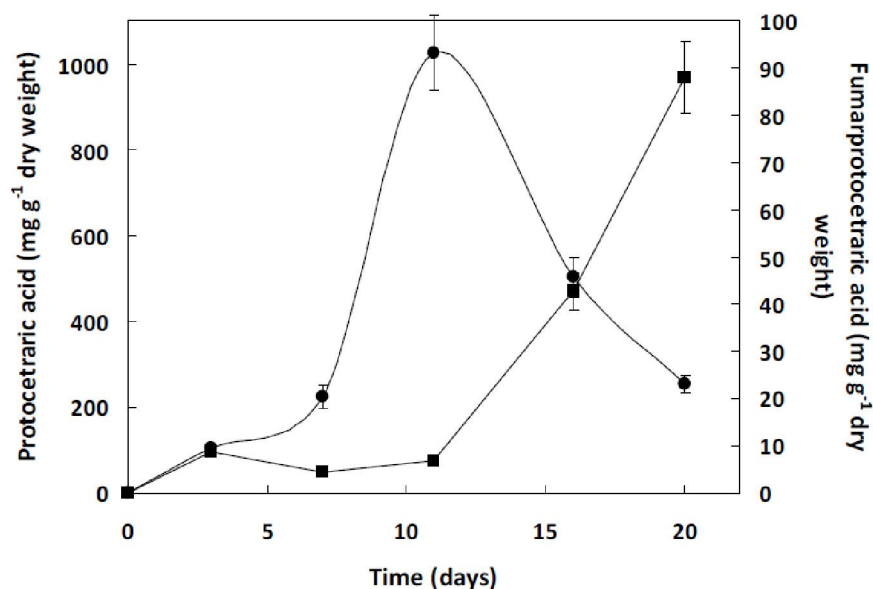


Figure 5 : Time-course of both protocetraric (●) and fumarprotocetraric (■) acids by alginate-immobilized mycobionts co-incubated with algal immobilisates. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols

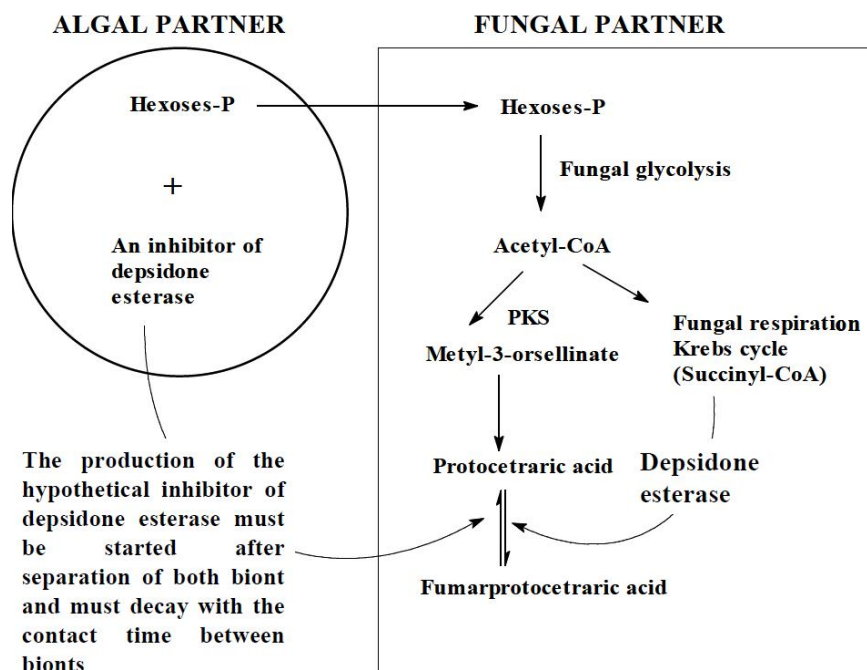


Figure 6 : Diagrammatic representation of the regulatory role of algal partner in the production and interconversion of *C. verticillaris* depsidones.

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transformation, possibly a small diffusible molecule produced by the chlorobiont. The production of this inhibitor declined with the time, since from the 11th, protocetraric acid begins to disappear and inversely, fumarprotocetraric acid begins to be actively synthesized. These conclusions have been summarized in the scheme of the Figure 6.

In the future, analysis and identification of metabolites secreted by algal immobilizates will be required in order to verify the proposed inhibition of the conversion of protocetraric acid into fumarprotocetraric acid.

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